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TECHNICAL MANUSCRIPT 229

IMMUNOLOGICAL STUDIES OF ANTHRAX: IV. EVALUATION OF THE IMMUNOGENICITY OF THREE COMPONENTS OF ANTHRAX TOXIN

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UNITED STATES ARMY
BIOLOGICAL LABORATORIES
FORT DETRICK

U.S. ARMY BIOLOGICAL LABORATORIES
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In conducting the research reported here, the investigators adhered to "Principles of Laboratory Animal Care" as established by the National Society for Medical Research.

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ABSTRACT

Three components of anthrax toxin (edema factor, protective antigen, and lethal factor) were separated and tested singly at three dose levels and in factorial combination (27 treatments) to determine their efficacy as immunogens in a resistant host (rat) and in a susceptible host (guinea pig). The efficacy of each component used as an immunogen was determined by its ability to (i) protect against the establishment of the disease, (ii) influence the number of bacilli-ml of blood at death, (iii) change the units of toxin/ml of blood at death, (iv) affect the antibody titer (Ouchterlony), and (v) alter the units of toxin neutralized/ml of blood. Lethal factor was the only immunogenic component against both B. anthracis spore and toxin challenge in each of the hosts tested. Protective antigen was protective in the guinea pig as both a primary and interactive response. In both animals the edema factor was nonimmunogenic by the methods of evaluation and gave poor protection against both the spore and toxin challenge; however, certain interactions were noted. The interaction of antibody and the dual defense mechanisms in resistant and susceptible hosts are discussed.

I. INTRODUCTION

The antigenic toxin of Bacillus anthracis has been shown to be composed of three components identified as Factors I, II, and III by English workers[1] and as edema factor (EF), protective antigen (PA) and lethal factor (LF) respectively by American workers.[2] "Protective antigen," as used currently in the literature on immunization against anthrax, is not the material given the same name earlier by Gladstone.[3] These components are of interest (i) as immunizing antigens changing either (a) the resistance of the host to challenge by the spore or vegetative cell or (b) the course of the disease once it is established, and (ii) as toxins that cause pathophysiological responses.

We[4] have recently summarized the development of the protective antigen of Wright and his colleagues and noted its use as an immunizing antigen for man. This development occurred even though the value of the other components of toxin in immunizing alone or in combination was not known, and in spite of the fact that certain strains of anthrax are "refractive" to immunity by PA.[5,6]

Recently, Stanley and Smith[1] reported on the degree of immunogenicity in guinea pigs of the three components and certain of their combinations. They concluded that Factor II immunized by itself. Factor I provided an additive effect, but the immunogenicity of Factor I plus II was decreased by the addition of Factor III.

The pathophysiological effects of the toxin components are only slightly understood. EF plus PA causes an edema when injected under the skin in guinea pigs; PA plus LF causes death of animals. The Fischer 344 rat is particularly sensitive in its reaction to whole toxin or the PA-LF combination. Nordberg et al.[7] have described pathophysiological changes occurring in the rabbit infected with anthrax, and Eckert and Bonventre[8] have shown similar responses in the rat caused by in vitro - produced toxin. We[4] have shown that several species of animals, when immunized, die with a lower number of bacilli and level of toxins in the terminal blood than do nonimmunized hosts. This suggests, among other possibilities, that increasing immunity to the establishment of the disease also increases the host's sensitivity to toxin. Ward et al.[6] also show that immunized guinea pigs die with a lower terminal number of bacilli/ml of blood than do nonimmunized animals. We[9] have shown also that the functions of resistance to establishment of disease and susceptibility to its course are individual functions. Immunity to anthrax, therefore, must be considered with respect to both factors, i.e., establishment as well as course of the disease. Of interest, too, is the development of basic information that may be used to develop an improved antiserum for use in treatment of anthrax.

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In the work reported here, we have considered the possibility that immunity may differ in different hosts and have attempted to evaluate in the resistant rat and the susceptible guinea pig the independent functions of resistance to establishment of disease (immunity in the classical sense) and of change of resistance in the course of the disease after its establishment. This evaluation used animals immunized to all the factorial combinations of the three components of toxin with the antigen at three dose levels. Rats were challenged with sterile toxins and both the rat and guinea pig were challenged with virulent spores. Five criteria used to evaluate the efficacy of the immunogens were (i) the antiserum titer in the blood as determined by the Ouchterlony technique, (ii) the units of toxin neutralized by antibodies in the blood, (iii) the immunity index that quantitates the increase in dose of spores required to cause an immunized host to give the same response in the same length of time as the nonimmunized host, (iv) the level of toxin in the blood at the time of death of the host challenged with spores and, (v) the number of bacilli in the terminal blood of the host challenged with spores.

II. MATERIALS AND METHODS

A. ANIMALS

Male Fischer 344 rats, two to four months old and weighing 200 to 300 grams, and Hartley guinea pigs from the Fort Detrick Animal Farm weighing 300 to 400 grams, were used.

B. CHALLENGE

A 1×10^9 spore culture of the highly virulent Vollum strain (V1b) of B. anthracis was used to challenge the guinea pig, and spores (1×10^9) plus egg-yolk medium[10] were used to challenge rats. Whole anthrax toxin containing 30 units/ml was used for the toxin challenge.[11]

C. PREPARATION OF TOXIN

The methods used for preparing anthrax toxins are described by Haines et al.[11] Casamino acids medium, 500 ml in each of eight Fernbach flasks, was inoculated with 2.5×10^8 spores of the avirulent Sterne strain of B. anthracis. Cultures were incubated statically at 37 C for 24 hours. After the cultures were pooled and centrifuged, all subsequent processing was carried out at 4 C.

D. FRACTIONATION OF WHOLE TOXIN

The toxic supernatant was separated into the LF, EF, and PA components. The method of Beall et al.[2] was used successfully in separating the PA and LF components. The eluates containing EF were combined and passed through a DEAE cellulose column to remove any traces of LF. The components were dialyzed against sterile distilled water at pH 7.5, dried under a vacuum of 10 to 30 microns of mercury for 18 to 24 hours in 2.5-ml quantities on an Aminco lyophilizer,* and stored at -25 C.

* American Instrument Co., Silver Spring, Maryland.

E. ASSAY ACTIVITY OF COMPONENTS

The agar diffusion method employed by Thorne and Belton[12] was used to quantitate antigenic activity of each component. Using a 1:8 dilution in gelatin phosphate of the Fort Detrick horse antiserum (DH-1-10A), the minimum amount of LF detectable was 226 μg dry wt equivalent to 25 μg protein. With this same antiserum the minimum amount of PA detectable was 137 μg dry wt equivalent to 17 μg protein. EF could not be detected by this method. Protein was determined by the method of Waddel.[13] Two separate preparations of horse antiserum (Sclavo-type produced by Instituta Serateraрico Tuscano, Sienna, Italy) yielded only one tube dilution difference in titers. When five times the minimal detectable amount of PA and LF were placed in adjacent wells single intersecting lines formed. However, the sensitivity of this technique does not preclude other antigens' being present in these preparations.

The biological activity of EF was titrated by the method of Smith et al.[14] EF (combined with PA) was the only component that gave a positive reaction in guinea pigs. The minimum amount of EF detectable by this method was 57 μg dry wt equivalent to 18 μg protein.

The biological activity of LF was determined by intravenous (iv) injection in Fischer 344 rats of 2.0 ml of a mixture consisting of one volume of LF component plus four volumes of PA. This ratio constituting 33.57 lethal units per 2 ml, killed the rats in 104 minutes.

F. TESTS FOR IMMUNOGENICITY

All animals were injected intraperitoneally (ip) with 0.1-ml amounts of components using the protocol described by DeArmon et al.[15] and Klein et al.[16] Three levels of each component (0, 100, and 1000 μg) were used. Each of the 27 factorial combinations of these three levels of each component was administered to each of 32 rats and 16 guinea pigs. All animals and all component combinations were randomized.

The level of immunity attained was described in terms of the immunity index of DeArmon et al.[15] This index represents the logarithmic difference in challenge dose required to cause the same time of response (death) in both immunized and control animals. The immunity index was computed for each animal by the equation:

$$I = \frac{1}{b}(X_1 - X_2)$$

where b is the slope of the dose response curve for the species of animals being considered, X_1 is the mean reciprocal time of response for the control, and X_2 is the mean reciprocal response time of immunized animals.

G. EXPERIMENTAL PROCEDURES

Two replications were performed for each type of challenge and for each host. The toxin-challenged rats provided three measures of response, (i) serum neutralizing potential, (ii) serum antibody titer, and (iii) time to death. The spore-challenged animals, both rats and guinea pigs, provided, in addition to the above measurements, two more measurements, (i) concentration of bacilli/ml of blood at death, and (ii) units of toxin/ml of terminal blood. One week following completion of the immunization protocol, 8 of each set of 32 rats and 12 of each set of 16 guinea pigs were bled by cardiac puncture. Approximately 1.2 ml of blood was withdrawn from each of the animals.

One-tenth of one ml of prechallenge serum from each of the blood samples was combined with 30 units of whole anthrax in vitro toxin as defined by Haines et al.[11] Each of the combinations (toxin plus serum) was injected iv into one assay rat and the time to death was recorded. The rat prechallenge serum was also tested on Ouchterlony diffusion plates for antibody titer, using parallel holes of alignment.

III. RESULTS

In the discussion of the data in this section all statistically significant treatments or interactions are mentioned.

A. RESISTANCE OF IMMUNIZED RATS TO TOXIN AND TO SPORE CHALLENGE

Regardless of whether rats were immunized with any of the three components alone or in combination, only the LF component protected against challenge with B. anthracis toxins. The response ratios and harmonic mean response times of these rats that died are shown in Table 1. The LF 100-μg treatment resulted in a high percentage of survivors. It was, therefore, not appropriate to calculate this statistic. However, the time to death of those responding (dying) was extended on the average to 46 minutes, a 66% increase in response time. The 1000-μg concentration saved 127 of 128 immunized animals from death following toxin challenge regardless of PA or EF treatments. The component PA by itself appeared to have a small effect in prolonging the response time of rats to toxin challenge but apparently is synergistic with LF, since 34 of 43 rats protected with 1000 μg of PA plus 100 μg of LF survived toxin challenge. The component EF gave essentially no protection. Thus, it was apparent that the LF component was highly immunogenic in rats against challenge with anthrax toxins.

TABLE 1. RESPONSE RATIOS AND HARMONIC MEAN RESPONSE TIMES OF IMMUNIZED RATS CHALLENGED WITH WHOLE TOXINS

EF, μg	LF, μg	Immunization Treatments PA 0 μg Ratio[a]	0 μg MTD,[b] min	100 μg Ratio	100 μg MTD, min	1000 μg Ratio	1000 μg MTD, min
0	0	16/16	70	15/15	73	15/15	85
0	100	15/15	78	14/14	73	4/12	-[c]
0	1000	0/15	-	0/14	-	0/15	-
100	0	14/14	74	15/15	69	8/15	-
100	100	13/13	73	14/15	82	2/16	-
100	1000	0/12	-	1/15	-	0/15	-
1000	0	12/12	73	15/15	72	9/14	-
1000	100	11/13	109	15/15	75	3/15	-
1000	1000	0/13	-	0/14	-	0/15	-

a. Number dead/number challenged.
b. Mean time to death, minutes.
c. No mean response times were calculated when 50% or more of the animals survived.

The analysis of variance for rats immunized similarly to those above but challenged by spores (Table 2) indicates that only LF had a highly significant effect, while EF interacted with LF significantly ($P<0.05$) to increase the immunity index. Of 60 surviving animals, 46 were immunized with LF. PA interacted antagonistically with LF ($P<0.05$).

B. IMMUNOGENICITY FOR GUINEA PIGS OF THE THREE ANTHRAX TOXIN COMPONENTS AGAINST B. ANTHRACIS SPORE CHALLENGE

The immunity indexes of the three components singly or in combination were calculated from the response time and are shown in Table 3. It is significant to note that again the LF component gave strong protection against the spore challenge, as did PA. Analysis indicates that the response to both of these components was statistically significant ($P<0.01$).

TABLE 2. HARMONIC MEAN RESPONSE TIMES AND IMMUNITY INDEXES OF IMMUNIZED RATS CHALLENGED WITH VIRULENT SPORES

Immunization Treatments							
		PA					
		0 μg		100 μg		1000 μg	
EF, μg	LF, μg	MTD,[a] hr	I[b]	MTD, hr	I	MTD, hr	I
0	0	16.4	0	14.9	-0.8	13.2	-1.8
0	100	14.8	-0.8	19.7	1.3	25.0	2.7
0	1000	19.5	1.2	21.1	1.7	16.0	-0.2
100	0	14.1	-1.2	14.6	-0.9	16.6	0.1
100	100	17.6	0.5	20.8	1.7	23.3	2.3
100	1000	23.3	2.3	17.2	0.4	24.1	2.5
1000	0	13.2	-1.9	18.5	0.9	28.5	3.3
1000	100	17.6	0.5	16.5	0.1	25.7	2.8
1000	1000	33.5	4.0	27.1	3.1	23.1	2.3

a. Mean time to death, hours.
b. Immunity index.

TABLE 3. HARMONIC MEAN RESPONSE TIMES AND IMMUNITY INDEXES OF IMMUNIZED GUINEA PIGS CHALLENGED WITH VIRULENT SPORES

Immunization Treatments							
		PA					
		0 μg		100 μg		1000 μg	
EF, μg	LF, μg	MTD,[a] hr	I[b]	MTD, hr	I	MTD, hr	I
0	0	26.3	0	25.4	-0.3	47.3	2.3
0	100	24.8	-0.5	29.9	1.1	39.6	3.0
0	1000	36.1	2.5	40.9	3.2	35.7	2.4
100	0	26.4	0.0	31.3	1.5	40.0	3.1
100	100	25.0	-0.5	27.7	0.5	40.2	3.1
100	1000	41.9	3.4	39.5	3.0	44.2	3.7
1000	0	22.5	-1.5	31.8	1.6	50.5	4.3
1000	100	25.5	-0.3	29.2	0.9	45.2	3.8
1000	1000	31.6	1.5	35.0	2.3	46.8	4.0

a. Mean time to death, hours.
b. Immunity index.

The marked interaction ($P<0.01$) between the LF and PA components is summarized in Table 4. PA and LF each had the strongest effect in the absence of the other. When 100 μg of either PA or LF is present, 100 μg of the other adds no beneficial effect, but 1000 μg of the other does extend the response time. The EF component neither had an immunogenic effect alone nor did it have an antagonistic effect on PA or LF. The data show that this interactive effect was opposite to that observed in rats.

C. NUMBER OF ORGANISMS AND UNITS OF TOXINS/ML OF TERMINAL BLOOD OF GUINEA PIGS AND RATS AS AFFECTED BY IMMUNIZATION

The terminal blood of rats and guinea pigs was assayed for number of viable organisms and concentration of toxins (Tables 5 and 6). The number of viable organisms in the terminal blood of guinea pigs (Table 5) was significantly lower ($P<0.01$) in animals immunized with both LF and PA. This effect, which was proportional to the amount of antigen given was consistent with other published data on anthrax infections in immunized animals.[4]

One hundred micrograms of EF increased the terminal concentration of organisms and of toxins, but the number of bacilli/ml and of toxins of terminal blood returned nearly to control level when 1000 μg of EF was used. This effect was not statistically significant.

TABLE 4. SUMMARY OF INTERACTIONS SHOWN IN TABLE 3 BETWEEN PA AND LF[a/]

LF, μg	Immunization Treatments PA 0 μg	PA 100 μg	PA 1000 μg
0	24.8	29.2	45.7
100	25.1	28.9	41.5
1000	36.1	38.3	41.8

a. EF had no effect so does not appear in this tabulation.

TABLE 5. GEOMETRIC MEAN CONCENTRATION OF ORGANISMS AND UNITS OF TOXINS IN THE TERMINAL BLOOD OF IMMUNIZED GUINEA PIGS CHALLENGED WITH VIRULENT SPORES

Immunization Treatments							
		PA					
		0 μg		100 μg		1000 μg	
EF, μg	LF, μg	10^8/ml	Toxins Units[a]	10^8/ml	Toxins Units	10^8/ml	Toxins Units
0	0	9.2	71	8.0	87	0.3	37
0	100	6.7	98	1.9	52	1.2	40
0	1000	1.3	15	0.3	17	3.5	51
100	0	11.4	106	1.4	46	3.5	85
100	100	3.3	69	1.5	64	1.4	60
100	1000	0.6	60	0.4	29	0.6	31
1000	0	8.8	88	2.2	72	1.3	70
1000	100	3.6	79	1.4	84	0.2	30
1000	1000	0.8	25	0.9	43	0.3	18

a. Concentration is expressed as potency units per ml of serum.

TABLE 6. GEOMETRIC MEAN CONCENTRATION OF ORGANISMS AND UNITS OF TOXINS IN THE TERMINAL BLOOD OF IMMUNIZED RATS CHALLENGED WITH VIRULENT SPORES

Immunization Treatments							
		PA					
		0 μg		100 μg		1000 μg	
EF, μg	LF, μg	10^6/ml	Toxins Units[a]	10^6/ml	Toxins Units	10^6/ml	Toxins Units
0	0	0.8	16.1	1.8	13.3	1.2	12.2
0	100	0.4	12.7	0.4	14.0	0.4	13.2
0	1000	0.2	11.9	0.2	14.3	1.3	14.8
100	0	2.8	13.9	3.0	14.4	0.6	14.7
100	100	2.6	13.5	1.6	14.4	0.2	14.2
100	1000	0.8	14.1	1.1	13.6	1.1	14.6
1000	0	0.8	15.0	5.6	13.3	1.3	14.4
1000	100	1.2	14.0	0.4	14.7	1.3	13.8
1000	1000	1.1	11.9	0.7	13.6	1.1	14.0

a. Concentrations are expressed as potency units per ml of serum.

The concentration of toxins in the terminal serum of guinea pigs is shown in Table 5. The effects of both PA and LF again are statistically significant ($P<0.01$) and similar to their effects on the terminal concentration of organisms. In addition, these two factors, PA and LF, interact ($P<0.05$) with each other, also PA with EF ($P<0.05$), and the interaction of all three was highly significant ($P<0.01$).

LF was the only component that caused any strongly significant ($P<0.01$) decrease in the terminal concentration of organisms in the rat (Table 6); however, PA interacted with EF as well as LF ($P<0.05$) to decrease the terminal organisms/ml of blood. The component EF showed the same pattern in this host as in the guinea pig, with an elevated count/ml associated with 100μg of this component with a return to control level when 1000μg was used ($P<0.05$).

In contrast to the results with guinea pigs, none of the treatments had any influence on the toxin concentration of the terminal blood of rats challenged with anthrax spores (Table 6).

The interaction among the terminal variables in the susceptible guinea pig and the resistant rat are graphically illustrated in Figure 1. In general, it appears that immunization extends the time to death, as evidenced by the Immunity Index, I, of both the susceptible and resistant animals. It also is apparent that as resistance attributable to immunity increases, the terminal number of organisms per milliliter of blood decreases in the guinea pig. Little or no influence in terminal numbers could be detected in the rat.

Dramatic changes were also noted in the relationship of terminal numbers of organisms to units of toxin in the guinea pig. As the number of bacilli per milliliter of blood increases so does the toxin concentration. The rat was unaffected in this relationship, which again points to inconsistencies in the pathophysiologic changes taking place in the immunized animal. The results on the guinea pig were consistent with those results published by Klein et al.,[17] Lincoln et al.[4] and Ward et al.[6]

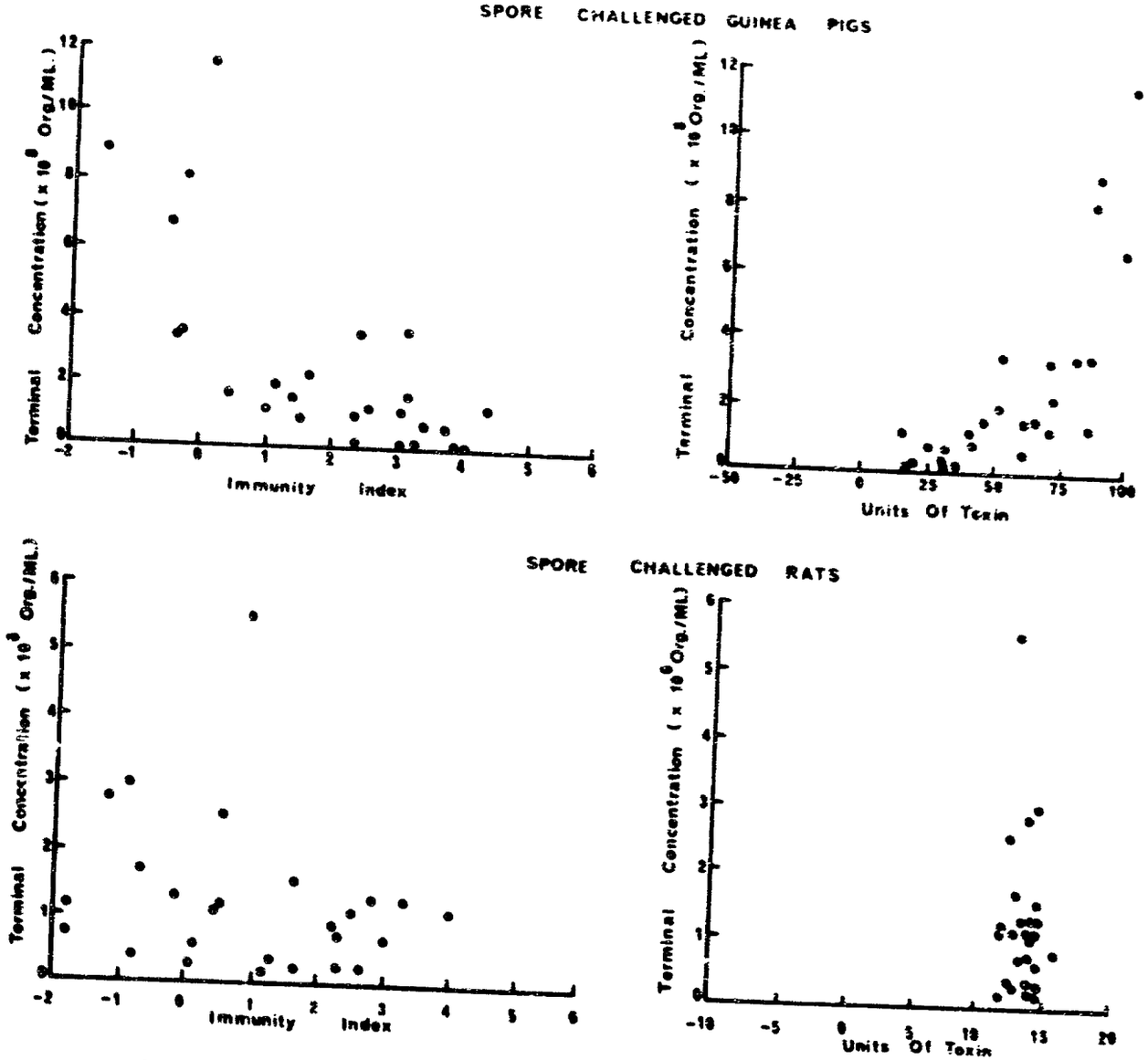

Figure 1. The Interaction Among the Terminal Variables in the Immunized Guinea Pig and Rat. Each data point represents one immunization protocol and is the mean of 9 to 10 animals distributed among two populations.

D. LACK OF DEMONSTRABLE IMMUNOGENICITY OF THE ANTHRAX TOXIN COMPONENTS AS MEASURED BY THE OUCHTERLONY TECHNIQUE AND SERUM-NEUTRALIZATION TESTS IN THE FISCHER RAT

The prechallenge sera from animals of both species were assayed for antibody on Ouchterlony plates. The sera from rats gave uniformly negative results to this assay. Sera of 47 of the 270 prechallenged guinea pigs showed a titer when assayed on Ouchterlony diffusion plates. The distribution of the positive-reacting sera, based on immunization protocol, is given in Table 7. The sera from guinea pigs immunized with EF produced no titer and therefore do not appear in this table. PA had little effect in producing precipitin lines that were demonstrable on Ouchterlony plates, but LF was much more consistently successful in producing identifiable lines against the antiserum used in these studies.

The neutralization tests of anthrax toxins by prechallenged blood serum are shown in Table 8 for the rat and guinea pig. Analysis of variance of the data revealed no significant toxin-neutralizing ability of any of the components or their combinations on the prechallenge sera from either species.

TABLE 7. DISTRIBUTION OF PRECHALLENGE SERA OF 47 GUINEA PIGS THAT SHOWED TITERS OF ONE-FOURTH OR GREATER FOLLOWING PA AND LF IMMUNIZATION[a]

Immunization Treatments			
	PA		
LF, μg	0 μg	100 μg	1000 μg
0	0	4	3
100	2	0	4
1000	11	13	10

a. EF did not have a significant effect so does not appear in this table.

IV. DISCUSSION

In this study we found that LF is the only component that is highly immunogenic against spore challenge of both hosts, the rat and the guinea pig, and toxin challenge of the rat. In the rat, addition of PA to LF increased the resistance significantly against the spore challenge but added nothing to the efficacy of the immunization against toxin challenge. In contrast, the guinea pig was effectively protected against spore challenge with either the LF or PA component and these components complemented each other.

Our past studies have drawn attention to the dual nature of resistance, with the resistant component being separated into factors operating during the establishment of anthrax in the host and those influencing the course of disease. The latter is especially evident during the terminal septicemic, toxemic phase of this disease. The results reported here again show the need for this phase separation.

TABLE 8. HARMONIC MEAN RESPONSE TIMES (MINUTES) OF ASSAY RATS CHALLENGED WITH 30 UNITS OF TOXIN COMBINED WITH 0.1 ML SERUM FROM IMMUNIZED RATS AND GUINEA PIGS

Immunization Treatments							
		PA					
		0 µg		100 µg		1000 µg	
EF, µg	LF, µg	Rat	Guinea Pig	Rat	Guinea Pig	Rat	Guinea Pig
0	0	162	198	145	165	183	160
0	100	155	201	151	170	127	136
0	1000	147	159	154	156	141	260
100	0	160	223	141	169	164	187
100	100	129	121	142	124	153	137
100	1000	157	163	175	180	198	197
1000	0	195	174	153	142	149	152
1000	100	144	139	167	134	154	245
1000	1000	185	234	138	208	184	211

The optimum concentration of antigen apparently is still greater than the amount used in this study and probably in any previous study. Even 1000 μg of antigen results in a relatively small proportion of the total immunity that will develop in a host in which live vaccine is used in conjunction with chemical antigens.[16] In earlier work we reported an Immunity Index (I) of eight, but in the present work the maximum index developed was four. Let us extrapolate somewhat directly from experimental hosts to man and consider the very low titers reported for humans.[18,19] One would surely conclude that larger quantities of antigen should be administered and indeed must be administered if high titers of antibody in the blood serum are to be developed. The optimum proportion of the antigens EF, PA, and LF would depend upon the criteria of immunity developed or those criteria judged most important. The combination of LF (1000 μg) plus PA (1000 μg) with EF ranging between 0 and 1000 μg was shown to be optimum, based on the individual or collective criteria of (i) immunity index evaluated by spore challenge (both hosts), (ii) survivors after challenge with anthrax toxin (only rats were tested), (iii) development and distribution of high titer (in guinea pigs but no titer developed in rats), and less conclusively (iv) the units of toxin neutralized by the postchallenge blood serum. Other immunization protocols would be selected if other criteria, such as an increased number of organisms in the blood, were used.

There is no experimental basis for selection or use of a single antigen for immunizing man or domestic animals. Gladstone's protective antigen likely was composed of all components of toxin; however, the "protective antigens" developed after Gladstone's work[20,23] are of undefined and different compositions. Some areas of discrepancy between our results and those of Stanley and Smith[1] are not unexpected because they (i) generated information only on the guinea pig in much more limited experimentation, and (ii) they used only one criterion, i.e., challenge with 1000 lethal doses of spores, to evaluate immunogenicity of the antigens. The authors of this paper definitely question the repeated statement by Smith[1,24] that the optimum proportions of the antigens cannot be determined because of the large number of animals required. Determination of the optimum proportions and concentration of antigens is a matter of desire and planning, since quantitative methods such as the immunity index[15] and modern designs combined with evaluation of multiple criteria of immunity, as we used in this work, allow the efficient acquisition of the necessary data.

The chief disagreement involves the apparent lack of effect of EF alone on resistance factors in our tests, although the British workers report a positive effect. Such differences may lie in the undetected contamination of one component with another in its active or toxoid form. The in vivo toxins may be expected to be complete and quite possibly other antigens of B. anthracis, particulate or soluble, are effective by themselves or interact with the toxic antigens. The fact, too, that Auerbach and Wright[5] observed

strain differences in regard to immunization with protective antigen shows the necessity for a broader basis of immunity than "protective antigen." It is suggested that until a much firmer experimental basis of immunization to anthrax is established, antigen containing at least LF and PA should be used for immunization of man. The observation made by Molner and Altenbern[26] may explain the response of the rat to PA. Their tests showed that PA disappeared from the blood between 90 and 120 minutes after injection, whereas LF disappeared sometime after 4 but before 24 hours. Persistence is usually considered a necessary characteristic of strong antigens and this observation may explain why the rat responds poorly to PA. At this time, we see little basis for eliminating EF, even though it has a slight antagonistic interaction with LF and PA, causing some decrease in immunity. In addition, Stanley and Smith[1] found that EF contributes to immunity. Clarification of this point by additional experimentation is necessary.

This work raises the question of what model may be used to extrapolate from experimental animals to man. We find the rat responds quite differently to these immunizing antigens than does the guinea pig. Again it seems that the conservative view on the development of an antigen for human and domestic animal use is use of the whole toxin.

Finally, again extrapolating from experimental host to man or domestic animals, in those cases where anthrax is established, immunization involves an unusual type of risk because of the diminished ability to detect anthrax in the blood stream. As the level of immunity is increased, the number of bacilli in the blood stream diminishes. In the present work, the level of decrease ranges from 5 to 30 times in the guinea pig and up to 4 times in the rat when immunized hosts are compared with nonimmunized ones. Certain treatments, usually those associated with EF at 100 μg, resulted in higher numbers of bacilli in the blood stream of the immunized host as compared with the control, usually with a decreased immunity index. The decreased level of bacteremia in immunized hosts is a general phenomenon. It is conceivable that immunized man and animals would die of anthrax without a detectable bacteremia if direct observation of blood for bacilli were used to identify anthrax. Based on the low titers reported for immunized man, there is no basis to predict whether this phenomenon should be observable in man or not; however, medical and veterinary practitioners may need to evaluate, among the alternatives presented for their consideration, the possibility that immunized hosts will have a greatly lowered level of bacteremia.

V. SUMMARY

In vitro - produced toxin of Bacillus anthracis was separated into its three components — edema factor (EF), protective antigen (PA), and lethal factor (LF). The efficacy of these components as immunogens was tested at 3 dose levels, (0, 100, and 1000 μg/dose) in all 27 factorial combinations of component levels. Hosts were the rat and the guinea pig, a resistant and a susceptible species, respectively. Both species were challenged with anthrax spores and, in addition, the rat was challenged with toxins.

The immunogenicity of each component was characterized in five ways (i) prior to challenge, in terms of (a) antibody titer and (b) units of toxins neutralized/ml of blood, and (ii) post-challenge by (a) increase in resistance to establishment of the disease, (b) number of bacilli/ml of terminal blood, and (c) units of toxin/ml of terminal blood.

These evaluations showed that (i) the LF component was highly immunogenic in rats against both toxins and spore challenge and in guinea pigs against spore challenge; (ii) the PA component was immunogenic against spore challenge in rats and guinea pigs, but completely ineffective against toxin challenge in rats; (iii) the EF component alone was nonimmunogenic; (iv) the effects of LF and PA were additive; and (v) EF added to LF, PA, or LF-PA combinations interacted significantly with LF to increase resistance in the rat, but was not additive in resistance in the guinea pig.

The number of organisms/ml of terminal blood decreased as resistance to establishment of disease increased. The units of toxin/ml of terminal blood was closely related to the number of bacilli/ml of blood at death.

Only 17% of the prechallenge serum of guinea pigs, principally among the LF treatments, produced antigen-antibody precipitin lines on Ouchterlony plates. The rat sera were all negative in this test.

The possibility of extrapolating these experimental results to effective immunization of man is discussed.

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